

# Primary structure of elongation factor 2 around the site of ADP-ribosylation is highly conserved from archaeobacteria to eukaryotes

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Elongation factor 2 (EF-2) from eukaryotes and archaeobacteria can be ADP-ribosylated by diphtheria toxin (DT) [(1977) *Annu. Rev. Biochem.* 46, 69–94; (1980) *Nature* 287, 250–251]. The primary structure of the ADP-ribose accepting region in EFs from the archaeobacteria *Thermoplasma acidophilum*, *Halobacterium cutirubrum* and *Methanococcus vannielli* was determined in order to elucidate the degree of conservation compared with 4 previously established eukaryotic sequences [(1971) *FEBS Lett.* 103, 253–255]. Within a 9-residue sequence including the site of ADP-ribosylation 5 positions were found to be occupied by the same amino acid in all the archaeobacterial and eukaryotic factors studied. There were more differences among the 3 archaeobacterial sequences than among the 4 eukaryotic ones.

<i>Archaeobacteria</i>	<i>Elongation factor</i>	<i>ADP-ribosylation site</i>	<i>Amino acid sequence</i>	<i>Evolution</i>
		<i>Diphtheria toxin</i>		

## 1. INTRODUCTION

Eukaryotic elongation factor 2 (EF-2) which catalyzes translocation of peptidyl-tRNA and mRNA on the ribosome, can be ADP-ribosylated by fragment A of diphtheria toxin (DT) [1], in contrast to the analogous prokaryotic factor EF-G [4]. The ADP-ribose accepting moiety of EF-2 is the novel, weakly basic amino acid 'diphthamide' isolated from yeast EF-2 and identified as a post-translationally modified histidine [5,6]. Digestion of EF-2 from phylogenetically distant eukaryotic sources yielded peptides with a conserved sequence over a stretch of 11 amino acids in which diphthamide occupied the position penultimate to the C-terminus [3,7,8].

Just like eukaryotes, all members of the archaeobacterial kingdom [9] so far studied contain a pro-

tein which can serve as substrate for the catalytic action of DT [2,10]. We isolated this protein and identified it as translocating elongation factor in 3 archaeobacterial species, the extremely halophilic *Halobacterium cutirubrum*, the methanogenic *Methanococcus vannielli* and the thermoacidophilic *Thermoplasma acidophilum* [11,12]. Amino acid analysis of the total postribosomal supernatant proteins from *H. halobium* grown with [<sup>3</sup>H]histidine provided evidence for diphthamide or its deamidation product diphthine being part of some halobacterial protein, presumably of an elongation factor [13].

To establish whether the ADP-ribose accepting sequence in eukaryotic EF-2 has an evolutionary precursor in common with the sequence in the archaeobacterial factors, we cleaved ADP-ribosyl-EF-2 from the 3 species mentioned, purified the labelled peptides and sequenced them. We found a remarkably high degree of conservation between archaeobacterial and eukaryotic sequences.

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## 2. EXPERIMENTAL

### 2.1. Partially purified elongation factors

EF-2 from *H. cutirubrum* Cz.Coll.M. 2088 was prepared as described [11]. Purification procedures for EF-2 from *M. vannielii* DSM 1224 and *T. acidophilum* DSM 1728 will be published elsewhere in detail; the main steps are summarized in the flow scheme (fig.1).

### 2.2. ADP-ribosylation

*H. cutirubrum*: 10 ml Tris-HCl buffer, pH 7.6, contained 5–20 nmol EF-2, 70  $\mu$ mol mercaptoethanol, 28.2 mmol KCl, 900 flocculation units of DT (Behring) preincubated with 12.5 mM mercaptoethanol, and 141 nmol [ $^{14}$ C]NAD (Amersham), spec. act. 4.84 mCi/mmol; incubation for

16 h at 37°C. With non-halophilic factors, 200 nmol KCl, 500 flocculation units of DT and 30 min incubation time were applied leaving the other conditions unchanged. After labelling protein was precipitated with trichloroacetic acid, washed and lyophilized.

### 2.3. Proteolytic cleavage

1 mg dried protein in 1 ml of 1% ammonium hydrogen carbonate was incubated with 50  $\mu$ g trypsin (Merck, TPCK-treated) for 5 h or longer periods of time (see section 3) at room temperature, or in 1 ml of 1% sodium hydrogen carbonate with 20  $\mu$ g proteinase Arg C (Boehringer) for 24 h at 37°C.

### 2.4. Affinity chromatography

After proteolysis the peptide mixture was applied to a column (10  $\times$  90 mm) of dihydroxyborylacrylamide (Affi-gel 601, Bio-Rad) equilibrated with 0.25 M ammonium acetate, pH 8.2, and washed with 30 ml of this buffer. The adsorbed material was eluted with 0.1 M ammonium acetate, pH 5.2.  $^{14}$ C-labelled fractions were collected and rechromatographed in the same manner or concentrated by freeze-drying, transferred into ammonium hydrogen carbonate by gel filtration (Sephadex G-15 or G-25, Pharmacia) and desalted by repeated freeze-drying.

### 2.5. Gel filtration

A Sephadex G-25 column (14  $\times$  60 mm) equilibrated with 0.01 M ammonium hydrogen carbonate was used.

### 2.6. High-performance liquid chromatography

The ADP-ribosyl peptides were further purified by reversed-phase chromatography on a Pep RPC 5/5 column, 5  $\times$  50 mm (Pharmacia FPLC system; for details see fig.2 legend); in several experiments, a Vydac TP RP10, 4.6  $\times$  250 mm (Chrompack), column was used in a Hewlett-Packard HPLC system.

### 2.7. Total amino acid analysis

The samples were hydrolyzed with 200  $\mu$ l of 5.7 M HCl at 110°C for 20 h. For determination a Biotronik amino acid analyser with a single column system was employed.

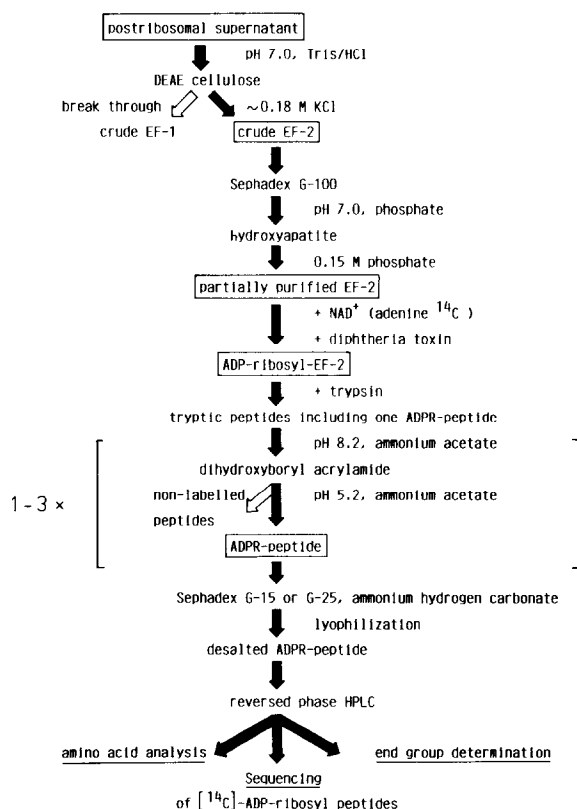


Fig.1. Flow scheme for the isolation of ADP-ribosylated peptides from non-halophilic archaeobacterial post-ribosomal supernatants. For *H. cutirubrum*, the peptide isolation procedure, beginning with 'ADP-ribosyl-EF-2', was the same; for EF-2 isolation see [11].

### 2.8. End group analysis

0.2–1.0 nmol of ADP-ribosyl peptide were dansylated [14] and hydrolyzed in 6 M HCl at 105°C for 16 h. Dansyl-amino acid analysis was performed by thin-layer chromatography [15].

### 2.9. Amino acid sequence analysis

For the Edman degradation [16] a prototype sequenator was used. Samples (about 2 nmol) were sequenced in the presence of 1 mg polybrene [17]. The phenylhydantoin derivatives of the amino acids were analyzed by an isocratic HPLC system [18].

### 2.10. Radioactivity measurement

A Packard Tri-Carb Liquid scintillation spectrometer was used. Labelled peptides were directly added to Quickscint 212 (Zinsser) and counted, radioactive proteins after precipitation on glass fiber filters.

## 3. RESULTS

After 3 repeats of the affinity chromatography procedure nearly pure ADP-ribosyl peptides were obtained from ADP-ribosyl-EF-2 digested with trypsin for 5 h and with arginine-specific protease for 24 h. When analyzing the factors from *T. acidophilum* and *H. cutirubrum*, high-performance liquid chromatography yielded only one major peak (fig.2, peaks A and B) containing about 75% of the  $^{14}\text{C}$  label applied to the column. Gel filtration on Sephadex G-25 indicated  $M_r$  values to be roughly 2000. End group analysis showed only dansyl-leucine. The two peptides had nearly the same amino acid composition except that serine found in the *Thermoplasma* peptide was replaced by alanine in *Halobacterium* (table 1). By automated Edman degradation the following sequences were established:

*T. acidophilum*: Leu-His-Glu-Asp-Ser-Ile-[gap]-Arg

*H. cutirubrum*: Leu-His-Glu-Asp-Ala-Ile-[gap]-Arg

For the penultimate positions of the sequences, the sequenator produced no amino acid derivative at all (as symbolized by 'gap') probably due to the high polarity of ADP-ribosyl-substituted diphthamide or diphthine.

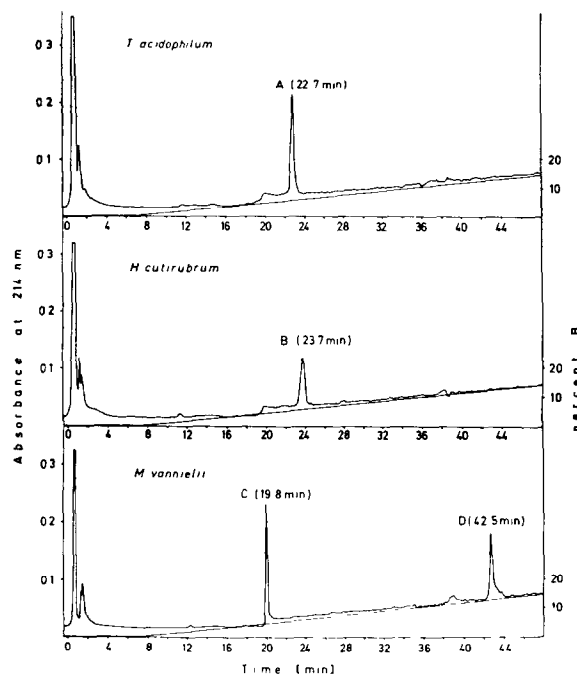


Fig.2. High-performance liquid chromatography of tryptic ADP-ribosyl peptides isolated with Affi-gel 601 (see section 2). Solvent A, water; solvent B, 0.1% trifluoroacetic acid in acetonitrile (v/v). A linear gradient (below) between 0 and 15% B in 45 min was employed; flow rate 0.7 ml/min. Detector adjusted to 0.5 absorption units full scale at 214 nm. Sample amount about 1.5–2 nmol each.

In the case of *Methanococcus* EF-2, incubation with trypsin for at least 18 h was required to obtain amounts of ADP-ribosylpeptide sufficient for analysis. The material eluted from the affinity column could be separated by gel filtration and by HPLC analysis into two peaks; the one running faster through Sephadex G-25 was eluted from the reversed-phase column after a much longer retention time than the other (fig.2, peaks C and D). Both peaks together contained about 75% of the added  $^{14}\text{C}$  label. As the time of digestion was increased the relative amount of the higher- $M_r$  compound decreased.

End group determination again showed leucine for peak D but histidine for C; amino acid analysis (table 1) indicated peptide D to be 6 residues longer than C corresponding with the results obtained by sequence analysis:

Table 1  
Amino acid composition of ADP-ribosyl peptides isolated by HPLC

Amino acid	Peptide from							
	<i>T. acidophilum</i> (fig.2, peak A)		<i>H. cutirubrum</i> (peak B)		<i>M. vannielii</i> (peak C)		<i>M. vannielii</i> (peak D)	
Asp	0.9	(1)	1.0	(1)	1.1	(1)	1.9	(2)
Thr	—	—	—	—	—	—	1.0	(1)
Ser	0.8	(1)	—	—	—	—	—	—
Glu	1.1	(1)	1.1	(1)	1.1	(1)	1.1	(1)
Gly <sup>a</sup>	0.7	—	1.1	—	0.9	—	1.0	—
Ala	—	—	0.9	(1)	0.9	(1)	1.7	(2)
Ile	0.6	(1)	0.7	(1)	0.8	(1)	1.3	(2)
Leu	0.9	(1)	0.8	(1)	—	—	1.0	(1)
Phe	—	—	—	—	—	—	0.9	(1)
His	0.9	(1)	0.9	(1)	0.9	(1)	1.0	(1)
Arg	0.9	(1)	0.9	(1)	0.8	(1)	0.9	(1)
Total <sup>b</sup>	7		7		6		12	

<sup>a</sup> Glycine is formed from adenine in the ADP-ribose side chain [3,19]

<sup>b</sup> The modified histidine residue is not included; an additional peak eluted between Phe and His probably represented diphthine [7]; the amido group of diphthamide and the ADP-ribose residue are split off under the conditions of acid hydrolysis [6]

Numbers represent mol residues per mol peptide as determined by amino acid analysis (20 h, hydrolysis without correction for destruction or incomplete release) and nearest integer

*M. vannielii*: His-Glu-Asp-Ala-Ile-[gap]-Arg  
(peptide C)

*M. vannielii*:  
(peptide D)  
Leu-Ile-Asp-Ala-Thr-Phe-His-Glu-Asp-Ala-Ile-[gap]-Arg

The differences in chromatographic retention times between the 4 peptides (fig.2) are in good agreement with the sequence differences.

#### 4. DISCUSSION

Isolation of ADP-ribosylpeptides from tryptic digests based on the affinity of the ribose moieties to dihydroxyboryl groups results in only one peptide thus giving evidence that the archaeobacterial elongation factors studied contain only one ADP-ribosylation site. This holds true also for the *Methanococcus* factor; of the two labelled peptides

(fig.2, peaks C and D) the smaller one (C) is obviously generated from the larger peptide by hydrolysis of a trypsin-sensitive phenylalanyl-histidine bond.

It seems highly probable that the ADP-ribosylated residue is in the penultimate position in which gaps appeared instead of phenylthiohydantoin amino acids during the sequencing procedure. The presumption that the novel amino acid diphthamide (or diphthine, see below) may be the ADP-ribose acceptor is corroborated by the sequence similarity between the ADP-ribose accepting sites of the 3 archaeobacteria and those of the eukaryotic factors so far studied [3] in which diphthamide occupies the corresponding position (fig.3).

Arginine-specific protease produced the same peptides from the *Halobacterium* and *Thermoplasma* factors as trypsin did; hence, before

<i>Thermoplasma</i> <i>acidophilum</i>	- Arg -	Leu - His -	Glu -	Asp -	Ser -	Ile -	Dip -	Arg
<i>Halobacterium</i> <i>cut-rubrum</i>	- Arg -	Leu - His -	Glu -	Asp -	Ala -	Ile -	Dip -	Arg
<i>Methanococcus</i> <i>vannielii</i>	- Leu - Ile -	Asp -	Ala -	Thr -	Phe -	His -	Glu -	Asp - Ala - Ile - Dip - Arg
yeast	- Ile - Leu -	Asp -	Val -	Thr -	Leu - His -	Ala -	Asp - Ala - Ile - Dip - Arg	
rat liver	- Val - His -	Asp -	Val -	Thr -	Leu - His -	Ala -	Asp - Ala - Ile - Dip - Arg	
bovine liver	- Val - His -	Asp -	Val -	Thr -	Leu - His -	Ala -	Asp - Ala - Ile - Dip - Arg	
wheat germ	- Val - Ser -	Asp -	Val -	Val -	Leu - His -	Thr -	Asp - Ala - Ile - Dip - Arg	

Fig.3. Primary structure at the sites of ADP-ribosylation in archaeobacterial EF-2s compared with eukaryotic EF-2s (eukaryotic sequences from [3]). Dip, diphthamide (clearly established for yeast) or diphthine.

cleavage the N-terminal leucine most likely had been preceded by arginine.

Within the 9-residue sequence which has been established in all the 7 factors analyzed (positions 5–13 in fig.3), 7 amino acids are conserved from *Halobacterium* and *Methanococcus* to eukaryotes, the *Thermoplasma* sequence showing one additional exchange in position 10. An invariable aspartate is located in that part of the eukaryotic and methanococcal sequences which is still unknown in the cases of *Thermoplasma* and *Halobacterium*.

Although the number of species and sequence positions compared is too small to draw definitive conclusions, there are, remarkably, a larger number of differences among the 3 archaeobacteria than between eukaryotes as phylogenetically distant as yeast and mammals. Only one amino acid, Glu in position 8, may be specific for the archaeobacterial sequences.

Considering a possible influence of the sequence differences on diphtheria toxin catalysis, the question arises as to whether the far slower rate of the toxin reaction with archaeobacterial factors than with eukaryotic ones [13,21] may be connected with the charged amino acids Glu (position 8) and Arg (position 5). Interestingly, *Methanococcus* EF-2 having Thr like the eukaryotic factors instead of Arg reacts somewhat faster with DT than *Thermoplasma* does (R. Gehrmann, unpublished). On the other hand, it is not yet established whether diphthine, as detected in *H. halobium* cell protein [13,22], or its amide, diphthamide, as found in eukaryotes, is the real ADP-ribose acceptor in archaeobacterial EF-2. Elongation factor from mutant eukaryotic cells containing diphthine was

ADP-ribosylated at a greatly reduced rate compared with wild-type EF-2 [22].

The physiological role of the diphthamide containing domain which may explain why its sequence has been conserved over at least two billion years is not definitely known, but there are indications that EF-2 serves as a substrate for a cellular ADP-ribosyltransferase acting in a similar way to diphtheria toxin [23,24]. Since the enzymatic effect of EF-2 is inhibited by ADP-ribosylation the domain common to eukaryotes and archaeobacteria may play a part in a novel translational regulation mechanism. Whether the conserved sequence may be directly involved in such a mechanism or is only needed as a recognition region for the diphthamide synthesizing enzymes remains to be clarified.

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